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## Kinetic Studies on the Destructive Action of Oxygen on Lyophilized *Serratia marcescens*

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### ABSTRACT

DEWALD, ROBERT R. (Fort Detrick, Frederick, Md.). Kinetic studies on the destructive action of oxygen on lyophilized *Serratia marcescens*. Appl. Microbiol. 14:568-572. 1966.—Dried *Serratia marcescens* (ATTC strain 14041) cells were exposed to various partial pressures of oxygen and nitrogen. The colony-forming ability of the organisms was rapidly destroyed during exposure to oxygen but was unimpaired by exposure to purified nitrogen. The degree of inactivation depended upon temperature, time, and the partial pressure of oxygen, regardless of whether pure oxygen or dry air was used. The inactivation by oxygen followed the expression  $-1nN/N_0 = k[O_2]^{1/3}t^{1/2}$ , where  $N_0$  and  $N$  are the number of viable organisms before and after exposure respectively,  $[O_2]$  is oxygen concentration,  $t$  is time, and  $k$  is the rate constant. At 25 C,  $k$  was  $276 \pm 36 \text{ moles}^{-1/3} \text{ cc}^{1/2} \text{ hr}^{-1/3}$  for oxygen pressures between 5.5 and 258 torr. In the temperature range between  $-78$  and  $40$  C, the rate constant may be expressed as  $k = 10^{5.95 \pm 0.2} \exp[(-430 \pm 26) \text{ cal/RT}] \text{ moles}^{-1/3} \text{ cc}^{1/2} \text{ hr}^{-1/2}$ .

Rogers (13) was one of the first investigators to recognize the lethal effects of oxygen on lyophilized organisms. Later, Naylor and Smith (12) reported results that substantiated Rogers' results. These investigators reported that survival was the highest for organisms stored under vacuum and lowest for those stored in air or oxygen. Atmospheres of nitrogen, hydrogen, and carbon dioxide yielded intermediate results. Scott (14) reported that the effect of the atmosphere upon the survival of dried bacteria depended upon the nature of the suspending medium and its moisture content. Recently, Lion and Bergmann (9, 10) listed numerous substances that protect lyophilized *Escherichia coli* against the lethal effects of oxygen. Lion (7) suggested that a prerequisite for effective protection against oxygen in the dry state is the accumulation of the solute around the bacteria, which he assumed to have occurred during lyophilization. Benedict et al. (1) reported that atmospheric oxygen killed 95% of dried *Serratia marcescens* cells in 10 min, that certain reducing agents prevented the action of the oxygen, and that humidity seemed to play no role in the phenomenon.

The nature of the adverse effect of oxygen on

dried bacteria is still not understood. Lion, Kirby-Smith, and Randolph (11) showed that free radical production, measured by an increase in the relative electron paramagnetic resonance (EPR) signal, occurred when dry *E. coli* was stored in the presence of oxygen. *S. marcescens* was shown by Dimmick, Heckly, and Hollis (3) to exhibit the same phenomenon. The EPR studies were extended by Heckly et al. (5) to include the effects of moisture, selected protective additives, and other environmental factors. Lion and Avi-Dor (8) showed that reduced nicotinamide adenine dinucleotide (NADH<sub>2</sub>) oxidase activity was inhibited in lyophilized *E. coli* after exposure to oxygen. Hess (6) recently reported that *S. marcescens* cells were rapidly inactivated when aerosolized in air but that their colony-forming ability was almost unimpaired when the organisms were aerosolized in relatively pure nitrogen.

This investigation was undertaken to determine (i) an expression for the inactivation of dried *S. marcescens* by oxygen, (ii) the Arrhenius parameters for inactivation, and (iii) whether the lethal effects of oxygen observed when water suspensions of *S. marcescens* are aerosolized (6) are similar to those observed when lyophilized organisms are exposed to oxygen.

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## MATERIALS AND METHODS

The methods for growing the *S. marcescens* strains (Fort Detrick strain 8UK, ATCC strain 14041), determination of viable-cell populations, lyophilization, and rehydration were described previously (2). Triply washed bacterial suspensions containing about  $2 \times 10^{10}$  organisms per milliliter were routinely used. From 45 to 70% of the viable-cell populations in the parent suspensions survived lyophilization, and these dried materials were used for the various studies.

The oxygen (Southern Oxygen Co., Hagerstown, Md.) used was predried by passing it through a trap of activated silica gel at Dry Ice temperatures. The dried oxygen was stored in 2-liter flasks on the high-vacuum manifold and used as needed. Room air was dried and stored in the same manner prior to use. Nitrogen containing less than 10 ppm of water and less than 2 ppm of oxygen was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., in sealed Pyrex bulbs. The nitrogen was further treated to reduce the oxygen content by allowing it to remain in contact with a mirror of vacuum-distilled sodium metal for a few days. Gas pressures were measured with a McLeod gauge or mercury manometer. Contact of mercury vapor with the test organisms was avoided by using cold traps.

The bacterial suspensions were lyophilized at pressures less than  $10^{-5}$  torr in 20-ml ampoules containing glass beads 4 mm in diameter. Immediately preceding exposure, the ampoules were slightly jarred to break up the dried caked material with the glass beads. At the beginning of this study, dried samples were vigorously shaken to determine whether this would increase the exposure surface of the dried material, thus resulting in a possible increase in the rate of inactivation during exposure. Vigorous shaking in the presence of the glass beads led to inactivation of a substantial percentage of the organisms, but with gentle jarring the mechanical destruction was negligible. In both cases, the remaining viable organisms decayed at the same rate when exposed to a given partial pressure of oxygen. Organisms dried in the absence of the glass beads, however, showed slower and nonreproducible inactivation rates during exposure. During the exposure studies at 25 C, ampoules containing the dried organisms were immersed in a water bath thermostatically controlled at 25 C ( $\pm 0.5$ ).

Dried organisms to be exposed to dry air at temperatures below 25 C were cooled to the desired temperature before the air was introduced. After the 1-hr exposure, the ampoules were evacuated and warmed to 25 C before rehydration.

## RESULTS

Some survival versus time data obtained by exposing lyophilized *S. marcescens* at various pressures of oxygen, dry air, and nitrogen are plotted in Fig. 1. No loss in viability could be detected when the dried organisms were held under vacuum (pressures less than  $10^{-5}$  torr) for periods up to 3 hr. The semilogarithm plot of  $N/N_0$  versus time indicates that the decay

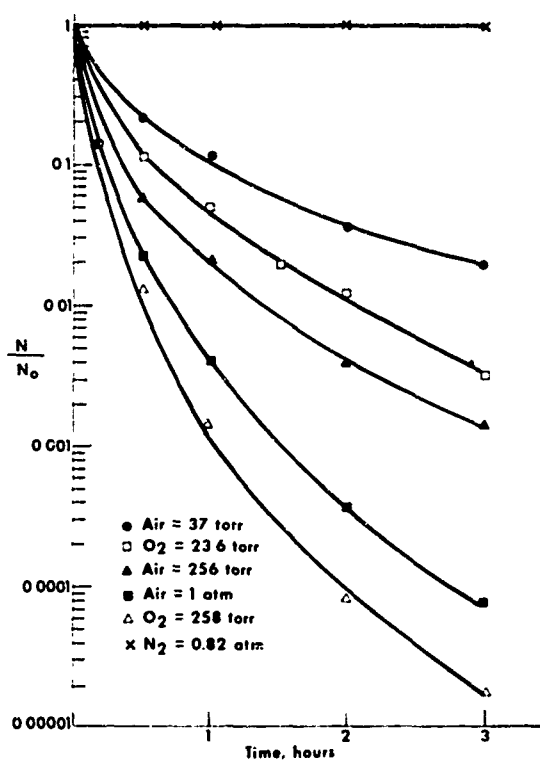


FIG. 1. Semilog plot of survival versus time after exposure of lyophilized *Serratia marcescens* to various pressures of oxygen, dry air, or purified nitrogen.  $N_0$  and  $N$  are the number of viable organisms before and after the exposure, respectively.

rate decreases as time increases for a given partial pressure of oxygen. Since no oxygen pressure changes were detected during these studies, it is concluded that the amount of oxygen required for inactivation is very small compared to the amount available in the system.

Figure 2 shows the dependence of viability upon the partial pressure of oxygen after 0.5- and 1-hr exposures at 25 C. The extreme sensitivity of the dried suspensions to low oxygen pressures was pronounced; about 75% of the organisms were inactivated in 0.5 hr by oxygen at a pressure of 10 torr. Also, losses in viability after exposure to dry air were identical to those obtained with pure oxygen when both were normalized to the same partial pressures of oxygen.

Figure 3 gives another representation of the inactivation curves given in Fig. 1. Plots of  $\log N/N_0$  versus  $(\text{time})^{1/2}$  fitted all the survival data well. It should be noted that the straight lines obtained for the various partial pressures of oxygen extrapolate to  $N/N_0 = 1$  at  $t = 0$ . A decay expression for the oxygen inactivation of lyophilized *S. marcescens* can be written:  $-\ln N/$

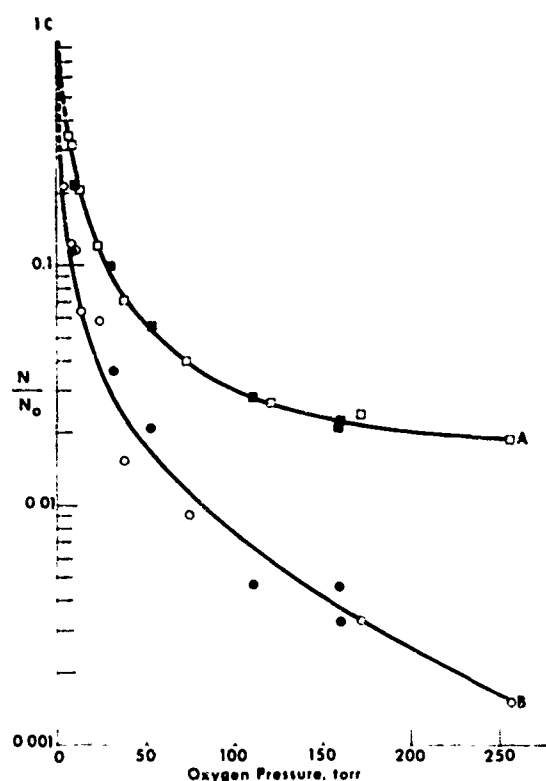


FIG. 2. Semilog plot of survival versus oxygen pressure after 0.5- (line A) and 1-hr (line B) exposures to oxygen or partial pressures of oxygen in dry air;  $N_0$  and  $N$  are the number of viable organisms before and after exposure respectively. Open circles and squares, pure oxygen; closed circles and squares, partial pressure of oxygen in dry air.

$N_0 = Kt^{1/2}$ , where  $K$  is a pseudo rate constant dependent upon the partial pressure of oxygen, and  $t$  is time. The pseudo rate constants,  $K$ , for all the oxygen inactivation data were obtained by determining the slopes of  $\log N/N_0$  versus  $(\text{time})^{1/2}$  plots by the least-squares method. These data are given in Table 1.

The pseudo rate constant is related to the oxygen concentration by the expression:

$$K = k[\text{O}_2]^n$$

or

$$\log_{10} K = n \log_{10} [\text{O}_2] + \log_{10} k$$

where  $n$  is the order of the rate expression in oxygen and  $k$  is the rate constant. A plot of  $\log_{10}$  of the pseudo rate constant,  $K$ , versus  $\log_{10}$  of the oxygen concentration is given in Fig. 4. A least-squares fit of the data yields a slope of  $0.328 \pm 0.022$ , which is approximately one-third. Therefore, the inactivation by the oxygen can be expressed as follows:

$$-1nN/N_0 = k[\text{O}_2]^{1/3}t^{1/2}$$

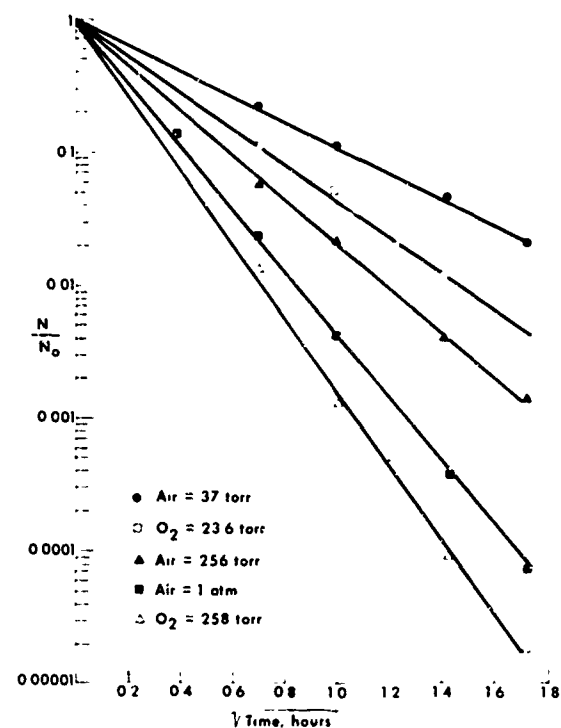


FIG. 3. Semilog plot of survival versus  $(\text{time})^{1/2}$  after exposure of lyophilized *Serratia marcescens* to various pressures of oxygen or dry air.  $N_0$  and  $N$  are the number of viable organisms before and after the exposure, respectively.

TABLE 1. Kinetic data for the inactivation of *Serratia marcescens* by oxygen

Oxygen pressure (torr)	Oxygen <sup>a</sup> concentration (moles/cc $\times 10^{-2}$ )	$K$ pseudo (hr <sup>-1/2</sup> )	$k$ (moles <sup>-1/3</sup> cc <sup>1/3</sup> hr <sup>-1/2</sup> )
258	139	6.51	271
172	92.5	5.50	261
160 <sup>b</sup>	86.1	5.17	252
159 <sup>b</sup>	85.5	6.13	300
121	65.0	5.27	283
110 <sup>b</sup>	59.2	4.79	275
71.6	38.5	4.15	264
53.7 <sup>b</sup>	28.9	3.85	271
37.7	20.3	4.28	337
30.8 <sup>b</sup>	16.6	3.02	258
23.6	12.7	3.31	306
11.0	5.92	2.11	251
8.0	4.30	1.85	245
7.8 <sup>b</sup>	4.20	2.29	306
5.5	2.96	1.78	267

<sup>a</sup> Calculated by using the ideal gas law.

<sup>b</sup> Dry air used as source of oxygen.

where  $N_0$  and  $N$  are the number of viable organisms before and after exposure, respectively,  $[\text{O}_2]$  is the oxygen concentration,  $t$  is time, and  $k$  is the rate constant, which was found to be  $276 \pm 36 \text{ moles}^{-1/3}\text{cc}^{1/3}\text{hr}^{-1/2}$  at 25 C.

Dried organisms at  $-78$ ,  $-15$ ,  $0$ , and  $40$  C were exposed to dry air at atmospheric pressure for 1 hr at these temperatures. The values obtained for the viability losses were corrected for the losses that occur when dried organisms under vacuum are subjected to the various temperatures for 1 hr (2). The corrected values of  $N/N_0$  were then used to estimate the rate constant,  $k$ , for the various temperatures. The results are plotted as the Arrhenius function in Fig. 5. A line determined by the least-squares method through the points for  $-78$  to  $40$  C yields:

$$k = 10^{5.95 \pm 0.42} \exp [(-430 \pm 26) \text{ cal/RT}] \text{ moles}^{-1/3} \text{ cc}^{1/3} \text{ hr}^{-1/2}$$

#### DISCUSSION

Lyophilized *S. marcescens* rapidly became nonviable when exposed to oxygen or dry air. The degree of this inactivation was found to be dependent upon time, temperature, and the partial pressure of oxygen. Lion and Bergmann (9) reported results on the inactivation of lyophilized *E. coli* by oxygen. Their qualitative results are in agreement with those of this work, but they did not report a decay expression for the inactivation process. However, their survival versus time data fit the  $\log N/N_0$  versus  $(\text{time})^{1/2}$  relationship. Dunklin and Puck (4) reported decay data of airborne organisms, and showed that the decay was more relative humidity (RH)-dependent in the presence than in the absence of added solutes. They considered the inactivation process as consisting of an initial rapid

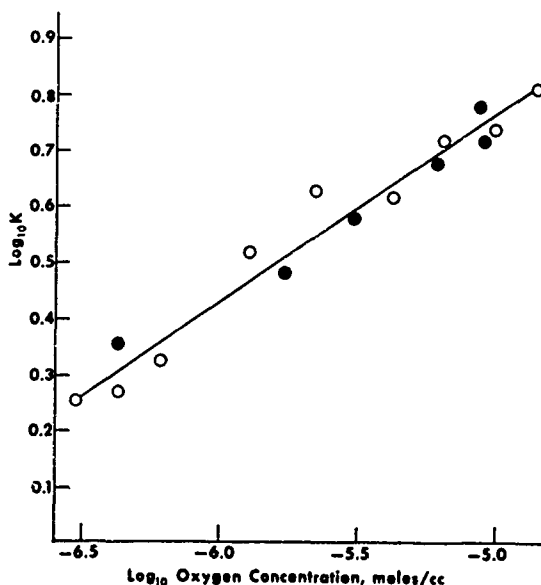


FIG. 4.  $\log_{10}$  of the pseudo rate constant,  $K$ , versus  $\log_{10}$  of the oxygen concentration. Open circles, pure oxygen; closed circles, dry air.

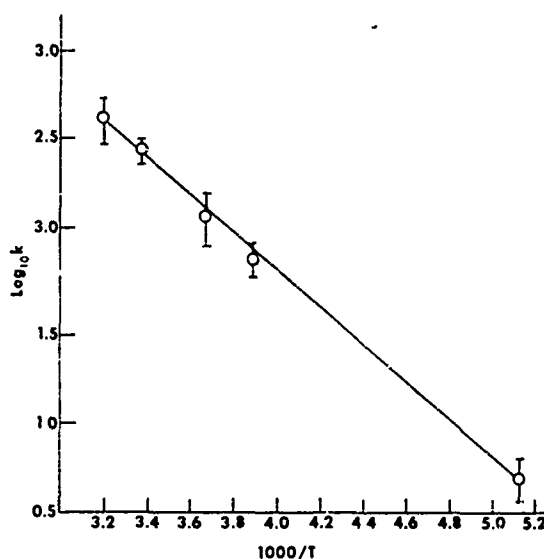


FIG. 5. Arrhenius plot for the inactivation of *Serratia marcescens*, by oxygen. On the abscissa,  $T$  is in degrees Kelvin.

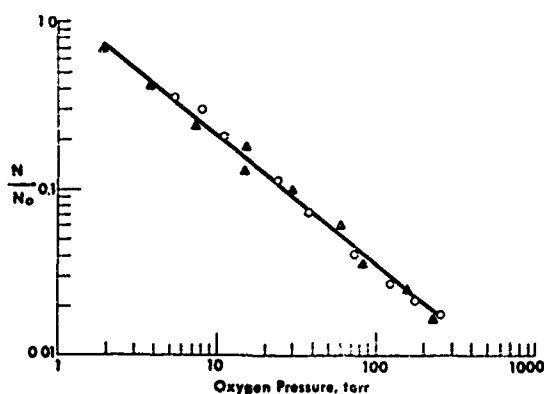


FIG. 6. Log-log plot of survival of *Serratia marcescens* versus partial pressure of oxygen.  $N_0$  and  $N$  are the number of viable organisms before and after the stress. Open circles, this work, lyophilized organisms after 0.5-hr exposure; closed triangles, Hess' data (6), aerosolized organisms after 32 min.

first-order rate process followed by a slower one. However, their data can be represented with equal success by a  $\log N/N_0$  versus  $(\text{time})^{1/2}$  plot, indicating that two different decay processes are not necessary to explain their data. Hess (6) recently reported a study on the inactivation of aerosolized *S. marcescens* in atmospheres containing various oxygen contents. Figure 6 gives a comparison of the inactivation reported by Hess (6) for aerosolized organisms with the results of the present work. There appears to be no marked difference in the degree of inactivation after 0.5 hr as a function of the partial pressure of oxygen in the two systems. It should be noted that Hess' data were generated at 40% RH, but

0% RH was used in this work; hence, direct comparison is not completely valid. However, preliminary studies conducted in this laboratory on the inactivation of lyophilized *S. marcescens* by oxygen in humidified air indicate that the degree of inactivation of washed lyophilized organisms was essentially independent of RH between 0 and 85%, but when organisms were lyophilized from suspensions containing 0.05% NaCl there was a marked RH dependence of the degree of inactivation by air. The results of these experiments will be reported elsewhere. Hess pointed out that the inactivation by oxygen was the major cause of death when *S. marcescens* were subjected to aerosolization in atmospheres of dehydrating levels of RH. Webb (15) suggested that the lethal effects in the aerosol are due to collapse of protein structures upon dehydration, and he later reported additives that were capable of replacing cellular water, thus increasing the survival of aerosolized *S. marcescens* (16). It appears that Webb overlooked the inactivation by oxygen; this is understandable when one considers the extreme sensitivity of dehydrated organisms to even small oxygen concentrations (Fig. 2).

Some remarks about kinetic interpretation of these data seem warranted at this time. The amount of oxygen required for inactivation is small, because no pressure changes were detected during the exposures (pressure changes >5% could have been detected). Lion, Kirby-Smith, and Randolph (11) came to a similar conclusion in their work on the inactivation of *E. coli* by molecular oxygen. The role played by molecular oxygen in the inactivation process is unknown, and it would be meaningless at this time to formulate a mechanism only on the basis of rate data. The preliminary kinetic studies reported in the present work, as well as those of Lion and Bergmann, show that the inactivation process is not simply pseudo first-order. In fact, the decay expression found here suggests that a chain mechanism involving radicals (5, 11) is probably occurring during the inactivation process.

It would be of interest to compare decay expression reported here with that of other atmospheric gases that impair viability. More kinetic data in this area should be useful in explaining the air sterilization of microorganisms.

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